U.S. v. Jiang
Docket No. 04-10128-MLV

Exhibit B.

Copies of the First Pages of Mr. Jiang's Four Publications.

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Electrochemical Desorption of Self-Assembled Monolayers Noninvasively Releases Patterned Cells from Geometrical Confinements

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This report describes a procedure based on the electrochemical desorption of self-assembled monolayers (SAMs) to release patterned mammalian cells from the constraints of these patterns. This procedure uses microcontact printing (μCP) and readily available thiols—$HS(CH_2)_{11}(OCH_2OCH_2)_3OH$ ($C_{11}EG_3$) and $HS(CH_2)_{17}CH_3$ (C_{18})—to confine cells into patterns; these methods are well established.[1,2] EG_3-terminated SAMs resist the adsorption of proteins (we call these surfaces "inert", meaning "inert to the adsorption of proteins").[3] Since mammalian cells attach to and spread on surfaces only if suitable extracellular matrix (ECM) proteins are present, EG_3-terminated SAMs also resist the attachment and spreading of cells.[1,4] Application of a cathodic potential of < -1.2 V (vs a stainless steel electrode) on gold caused the SAMs to desorb.[5,6] Removal of EG_3-terminated SAMs from a gold surface allowed the surface to adsorb proteins.[7] Here we have used μCP to pattern SAMs and electrochemistry to desorb the confining $(EG)_3$-terminated SAM; this procedure allows cells to be grown in patterns and then to be released from these patterns. After desorption of the $(EG)_3$-terminated SAM, cells can attach to, and spread across, previously inert areas (Figure 1).

We used electrochemical desorption of EG_3-terminated SAMs to demonstrate that bovine capillary endothelial (BCE) cells confined to microislands of patterned SAMs can be released from their confinements. Patterning of BCE cells was accomplished by methods previously described,[8] with C_{18} as the protein-adsorbing SAM and $C_{11}EG_3$ as the inert SAM (see Supporting Information for more details).[9] Cells were confined on these micropatterns in normal growth media for 24 h. After application of a cathodic voltage pulse (< -1.2 V, vs stainless steel, for 30 s), cells began to spread perceptibly from the microislands (Figure 1). We believe that the voltage pulse desorbed some or all of the $(EG)_3$-terminated SAM and that ECM proteins such as fibronectin (FN) present in the fluid medium or secreted by cells rapidly adsorbed onto regions that had been previously rendered inert by these SAMs. The cells migrated across the entire surface as if they were migrating on "bare" gold (that is, gold with no SAM but with a layer of proteins adsorbed from the medium). They also underwent normal growth and proceeded to cytokinesis on these substrates.

We quantified the amount of proteins adsorbed on the gold surfaces after the voltage pulse by using surface plasmon resonance (SPR).[10] Figure 2 compares the adsorption of serum proteins from the growth medium onto substrates initially coated with $C_{11}EG_3$ that underwent the cathodic pulses (ranging from -1.0 to -1.8 V, for 30 s). SPR measured the increase in the thickness of the thin organic film (SAMs or proteins) on the gold; this increase was reflected in the change in response units (ΔRU). ΔRU was mainly caused by the adsorption of proteins when the growth medium for
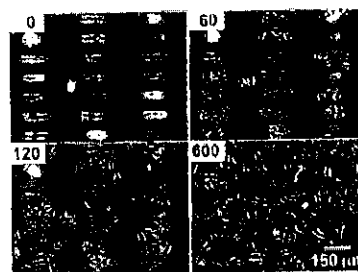
Figure 1. BCE cells were allowed to attach to a surface patterned with $C_{11}EG_3$ and C_{18}. Application of a cathodic voltage pulse (-1.2 V for 30 s in this case) released the cells from the microislands. The numbers indicate the time elapsed (in minutes) after the voltage pulse.
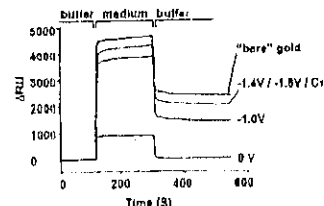
Figure 2. SPR sensorgrams compare the amounts of adsorbed proteins on the SAMs originally coated with $C_{11}EG_3$ that underwent various cathodic voltages. The sensorgram on the SAM formed by C_{18} is also shown. "Bare" gold is a gold surface that does not have a SAM.

BCE cells was allowed to flow over the gold substrate.[11] The sensograms indicate that the gold surface ceased to be inert after a voltage pulse of -1.0 V and became similar to a SAM of C_{18} in its ability to adsorb proteins after pulses of -1.4 and -1.8 V. By using the ΔRU on the SAM formed by C_{18} as a complete monolayer of serum proteins, we estimated the coverage of proteins on SAMs-coated gold to be 56% after -1.0 V, and 100% after -1.4 (or -1.8 V).[12] A bare gold surface seemed to adsorb slightly more proteins than a SAM of C_{18}, but the difference in optical characteristics of these surfaces made a direct comparison difficult.[13] Cyclic voltammetry also corroborated the desorption of $C_{11}EG_3$ (see Supporting Information, Figure 1).

Proteins adsorbed on a SAM formed by C_{18} did not desorb after application of voltage pulses in the range used here. Quantitative fluorescence measurements on rhodamine-labeled FN adsorbed on the C_{18}-covered area of the substrate showed indistinguishable values before and after the voltage pulse. This result agrees with a recent report that similar cathodic potentials did not decrease the quantity of plasma proteins that adsorbed onto gold surfaces.[14]

BCE cells appeared to be intact and normal after the voltage pulse. The maximum voltage gradient was 180 mV/mm (1.8 V across a distance of 10 mm between the two electrodes) in our experiment; this voltage gradient is comparable to the voltage

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Directing cell migration with asymmetric micropatterns

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This report shows that the direction of polarization of attached mammalian cells determines the direction in which they move. Surfaces micropatterned with appropriately functionalized self-assembled monolayers constrain individual cells to asymmetric geometries (for example, a teardrop); these geometries polarize the morphology of the cell. After electrochemical desorption of the self-assembled monolayers removes these constraints and allows the cells to move across the surface, they move toward their blunt ends.

motility | polarity | self-assembled monolayers

This report demonstrates that imposed polarity of an adherent mammalian cell, that is, its morphology as characterized by a wide front (typically the blunt end) and a narrow rear (typically the sharp end), determines its direction of motility (1, 2). We patterned self-assembled monolayers (SAMs) on gold to confine single cells initially to polarized shapes (3, 4). A brief pulse of voltage applied to the gold released the cells from their constraints and allowed them to move freely across the surface (4). The initial direction of motility of cells correlated with the polarity of their original shape; we conclude that polarization of the shape of cells is sufficient to determine their directions of motion.

The migration of mammalian cells typically includes the following processes: (*i*) morphological polarization (characterized by a wide front and a narrow rear); (*ii*) extension of membranes toward the direction of motility; (*iii*) formation of attachments between these leading membranes and the substrate; (*iv*) movement of the bulk of the cell body; and (*v*) release of attachments from the substrate at the sharp end (2). These processes together result in net translocation of the cell body (Fig. 1A). Many types of motile mammalian cells adopt a "teardrop" shape with a wide leading edge (dominated by structures termed the lamellipodia) that extends in the front and a narrow tail that releases and retracts. Most types of cells can polarize and move without stimuli (2, 5). Under the influence of a stimulus (chemical or mechanical), cells can polarize and move directionally (toward or away from the stimulus). It is not clear, however, whether morphological polarity of the cell itself can determine the direction of motility. We addressed this uncertainty by defining the polarity of adherent cells using an asymmetrically patterned substrate without a gradient of stimulant. We then released the constraint on the shape and location of the cells and assessed the direction of motility for individual cells. This approach is, to our knowledge, the first test of the hypothesis that the shape of a cell determines the direction of its motion. Other parameters that characterize the motion of cells, such as their speed and their tendency to make turns, are not affected by the initial constraints.

Recently, Parker et al. (6) showed that cells, when confined to a square shape, in the absence of gradients of stimulant, preferentially extended their lamellipodia from the corners (Fig. 1B). Because the confinement is static in their work, there was no net translocation of the cell body. Further experiments with various shapes showed that lamellipodia of several types of cells
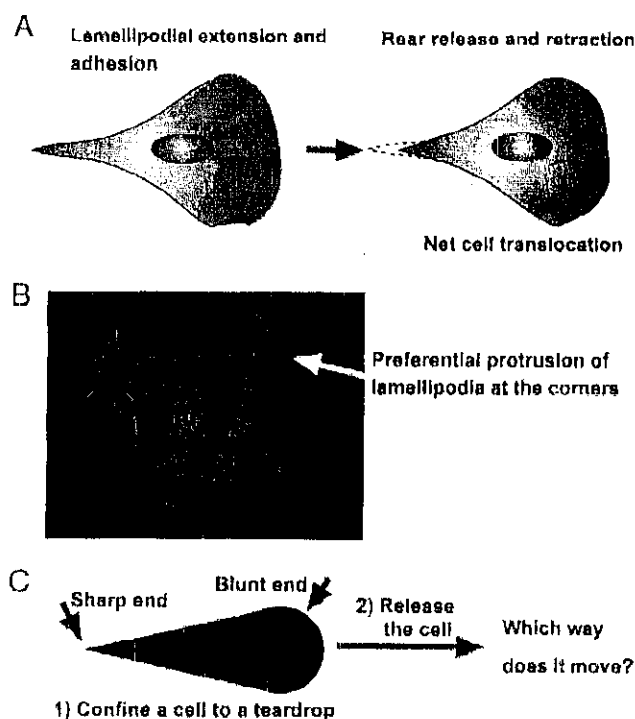

Fig. 1. A problem on cell motility. (A) A cartoon illustration of the migration of a typical mammalian cell on a flat surface. This teardrop shape is found in many types of cells. (B) Cells confined to squares preferentially extend their lamellipodia from the corners. nu, nucleus. (C) If a cell is confined to a shape of teardrop, will the cell preferentially extend its lamellipodia from the sharp end or from the blunt end? If released from confinement, in which direction will it likely move?

were more likely to protrude from sharp corners than from corners with larger angles (7).

The natural shape (often approximately a teardrop) of a motile cell and the preferential protrusion of lamellipodia from the corners of a square, stationary cell seem contradictory. For a cell confined to a teardrop shape, the results of Parker et al. (6) suggest that a cell would extend its lamellipodia from the sharp corner and that, once released, the cell would move toward the sharp end of the drop; the natural morphology of moving cells suggests that a released cell would move toward its blunt end (Fig. 1A) (2).

We have developed a technique that uses patterned SAMs of alkanethiols on gold to confine a cell initially to an arbitrary

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A Miniaturized, Parallel, Serially Diluted Immunoassay for Analyzing Multiple Antigens

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This report describes a miniaturized, microfluidic version of a serial-dilution fluorescent immunoassay.[1] This assay is capable of analyzing multiple antibodies quantitatively and in parallel in small volumes (<1 μL) of liquids in one experiment. It uses a network of microfluidic channels to achieve serial dilution; we call this system of microchannels a microdilutor network (μDN, Figure 1). The branching structures of the μDN serially dilute one stream with a second (so long as proper mixing occurs at each stage). Flow in microchannels is normally laminar;[2] to ensure complete mixing, we incorporate chaotic advective mixers (CAMs) into the μDN.[3] In this assay, we use this device to dilute a sample serially with buffer.

We illustrate this assay by determining the concentrations of antibodies (IgGs in this case) in HIV+ human serum (anti-gp41 and anti-gp120). In the assay, serially diluted solutions of serum flow in channels across orthogonal, parallel strips of HIV ENV proteins (antigens, gp41, and pg120) adsorbed on a polycarbonate membrane. The soluble antibodies bind to these adsorbed antigens and are themselves immobilized; the quantity of adsorbed antibody can be measured using a second, fluorescent antibody.

We believe that this method provides a new and general approach to one of the most common bioanalytical procedures.[1] The μDN replaces the set of microwells used in manual serial dilutions; the format of this assay is the same as that of the traditional ELISA-type assays for HIV; this format includes the sequential adsorption and immobilization of antigen, antibody, and secondary antibody.[4] This method is, we believe, generalizable to the analysis of 10–100 antibodies (as long as there is no cross-reactivity between these antibodies), although we have analyzed only two.

The microfluidic device has two components (Figure 1): the first component is the μDN that dilutes the analyte (serum containing HIV antibodies) serially using CAMs (the top portion of Figure 1) to achieve mixing; the second component is a membrane that presents stripes of immobilized proteins (in this case, gp41 and gp120; bottom portion of Figure 1 and Supporting Information Figure S1). The dilutor mixes and dilutes the serum with a buffer containing 5% bovine serum albumin (BSA), used to block nonspecific adsorption of antibodies on the surfaces of the device); this procedure generates a series of solutions containing exponentially decreasing concentrations of antibodies. The design in Figure 1 achieves 1:1 mixing of serum-containing solution and buffer in each stage of dilution. Each stage thus decreases the concentration of antibodies by one-half. (Supporting Information Figure S2 quantifies the efficiency of mixing in the device.) Using 1:1 dilutions and 10 sequential stages of mixing and dilution, we have achieved a dynamic range of $2^{10} \sim 10^3$. A 1:3 dilution would, in principle, achieve a dynamic range of 10^6 with the same number of stages of mixing and splitting; we have not yet demonstrated this range experimentally. We demonstrated the function of the dilutor using BSA conjugated to fluorescein (BSA-FITC) and
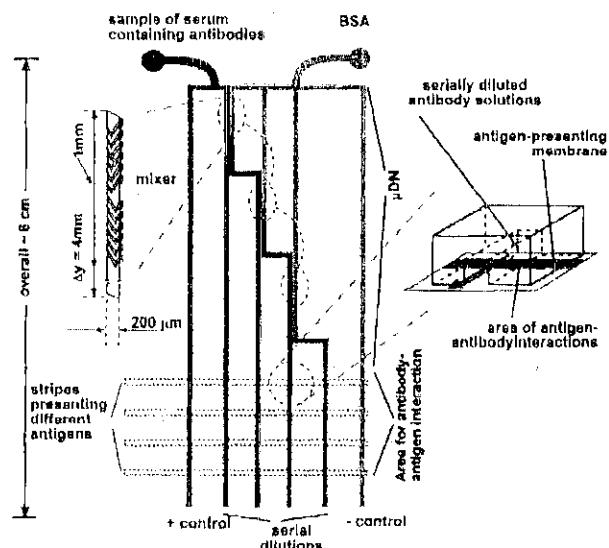
Figure 1. Schematic representation of the analytical device. The μDN (top of figure) generates a serial dilution of analytes that cross over immobilized antigens. Each CAM includes four cycles of herringbone patterns and is 4 mm long; one cycle is shown in an expanded view. The bottom part of the figure suggests how the serially diluted antibody interacts with spatially segregated antigens, immobilized on a polycarbonate membrane.
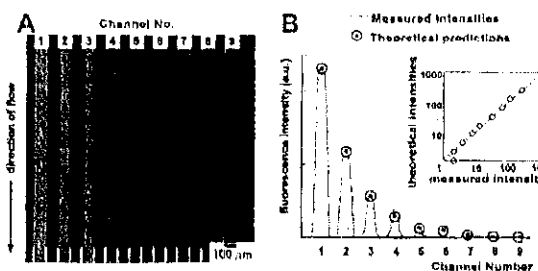
Figure 2. The on-chip dilutor serially dilutes BSA-FITC. (A) A serial dilution network that dilutes a total of nine channels, where a 1:1 mixing is achieved serially. (B) The graph shows the measured fluorescent intensities in all nine channels (indicated on the x axis). The insert shows the correlation between measured and calculated intensities across a dynamic range of almost 10^3.

phosphate buffer saline (PBS) in solution (Figure 2); observed and expected values agree well.

The polycarbonate membrane (the second component of the system) presented the antigens patterned in microstripes (bottom portion of Figure 1, Supporting Information Figure S1A and C; assays based on similar principles have been demonstrated previously[5,6]). We used an array of microchannels to deliver gp120 and gp41 to a polycarbonate membrane.[5] The membrane contained pores of diameter ~200 nm; these pores presented large surface areas

Palladium as a Substrate for Self-Assembled Monolayers Used in Biotechnology

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This paper describes self-assembled monolayers (SAMs) on palladium that resist the nonspecific adsorption of proteins and the adhesion of mammalian cells. These SAMs form when thin films of palladium are exposed to solutions of alkanethiol with the general structure $HS(CH_2)_m(OCH_2CH_2)_nOH$ ($m = 2, 11; n = 3, 6, 7$). Ellipsometry and surface plasmon resonance spectroscopy (using a palladium-on-gold substrate) showed that these SAMs resist adsorption of all proteins present in bovine serum. Microislands of SAMs of octadecanethiol on palladium allowed patterned adhesion and growth of mammalian cells (in a "sea" of oligo(ethyleneglycol)-terminated SAM). The oligo(ethyleneglycol)-terminated SAM resisted the invasion of cells for over four weeks under standard conditions of cell culture; similar SAMs on gold remained patterned for only two weeks.

This report describes self-assembled monolayers (SAMs) formed from oligo(ethyleneglycol) (OEG)-terminated alkanethiols on palladium and demonstrates that these SAMs resist nonspecific adsorption of proteins and adhesion of mammalian cells. (We call surfaces that resist adsorption of proteins and adhesion of cells "inert surfaces".) Most OEG-terminated SAMs on gold and silver are inert.[1−3] We and others have used these inert surfaces to study the interactions between ligands and receptors,[4−7] to investigate adsorption of proteins on surfaces,[8,9] to control the adhesion of mammalian cells,[10−14] and to fabricate surfaces that resist bacterial adhesion.[15,16] Tools based on inert SAMs allow control of adhesion of cells and modulation of the shape and size of cells using well-defined patterns of ligands on surfaces. These tools facilitate the investigation of interactions between cells and surfaces and the study of cell biology.[3,10,17−20]

Thiols also form well-ordered SAMs on thin films of palladium.[21] In this paper, we show that OEG-terminated SAMs on palladium provide inert surfaces useful for culturing mammalian cells. One specific OEG-terminated SAM on palladium performed better than any OEG-terminated SAM on gold. This SAM on palladium remained inert for at least 4 weeks under conditions of cell culture; analogous SAMs on gold lasted for 2 weeks under the same conditions. The long-term inertness of OEG-terminated SAMs and the low toxicity of palladium toward cells make SAMs on palladium excellent candidates for use in long-term culture of patterned cells.

EXPERIMENTAL SECTION

Reagents and Materials. All antibodies and reagents were obtained from Sigma (www.sigmaaldrich.com) unless otherwise specified. $HS(CH_2)_{11}(OCH_2CH_2)_3OH$, **1**, and $HS(CH_2)_{11}(OCH_2CH_2)_6OH$, **2**, were from Prochimia (www.prochimia.com), and $HS(CH_2)_2(OCH_2CH_2)_7OH$, **3**, was from LLC engineering (www.chemsupply.ch). All reagents were used as received. The molecular structures of these thiols were confirmed by NMR and mass spectrometry.

Preparation of Substrates and Ellipsometry. We evaporated 10−200-nm-thick films of palladium onto titanium- or chromium-primed substrates of glass or silicon wafers. Immersing the palladium films in 2 mM solutions of thiol in ethanol for 2−55 h yielded the SAMs.[21] After incubating the substrates in fibroblast growth medium (Invitrogen, www.invitrogen.com) at 25 °C for 12 h, we used ellipsometry (using a VB-400 ellipsometer and a HS 190 monochromator from J. A. Woollam Co., www.jawoollam.com) to determine the thickness of the dielectric layer (and, hence, the amount of adsorbed proteins) on each type of

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